

REMARKS

After entry of this amendment, claims 1 and 4-22 are pending. Claims 2 and 3 have been cancelled without prejudice or disclaimer. The claims have been amended without prejudice and disclaimer and find support *inter alia* in the original claims. Because the amendments to claim 1 and 4 are requested by the Examiner and based on an election of species, Applicants reserve all rights to rejoinder and examination of the non-elected species upon allowance of the generic claims or the claims directed to the elected species. 37 CFR § 1.141; MPEP § 809.02(a). The amendments to claim 1 further find support in the specification at page 7, lines 16, 20, and at page 8, lines 18-20. The amendments to claim 12 further find support in the specification at page 17, lines 7-41, page 32, lines 4-30. Claims 18 and 19 have been amended without disclaimer or prejudice for clarification. New claims 21 and 22 find support in the specification at page 9, lines 32-34, page 10, lines 6-12, and page 53, lines 15-17. The new claims are consistent with the restriction requirement. No new matter has been added.

The specification has been amended to include a paragraph directed to the related applications already of record and to delete a paragraph added in the preliminary amendment. The amendment to page 47 which corrects a typographical error finds support in the sequence listing. No new matter has been added.

Election/Restriction

The Examiner stated that the election for R2 and R3 was “unsaturated C2-C4-alkylcarbonyl.” (See page 2, item 1). Applicants respectfully disagree; the provisional election was rather to “unsaturated C2-C~~24~~-alkylcarbonyl.”

The Examiner treated the election as an election without traverse. Applicants respectfully disagree that the election should be treated as being without traverse. In the response to the restriction requirement, Applicants stated that the election was a provisional election with traverse and that “there is no undue burden on the Examiner to search this invention.” (See Response to Restriction Requirement dated November 2, 2007, page 1, first paragraph, last sentence). Pursuant to 37 CFR §1.143, Applicants clearly gave reasons for the

traverse. Furthermore, as stated in § 803 of the M.P.E.P. “[i]f the search and examination of the entire application can be made without serious burden, the examiner must examine it on the merits, even though it includes claims to independent or distinct inventions.” (M.P.E.P. § 803). Because Applicants gave sufficient reasons for traversing the restriction, Applicants respectfully request that the election be considered as having been made with traverse.

Claim Objections

The claims have been amended to reflect some of the elected species. Because the restriction was based on an election of species, Applicants will amend the claims in due course if needed once allowable generic and/or elected subject matter is otherwise indicated. See 37 CFR§1.111(b).

Rejections under 35 U.S.C. § 112, second paragraph

The Examiner rejects claims 1-20 under 35 USC § 112, second paragraph, as being indefinite. Specifically, the Examiner rejects claim 1, and claims 2-20 dependent thereon, as indefinite for reciting “or a radical of general Formula II”, alleging that it is unclear whether this phrase is intended as an alternative to Formula I or as a choice for R¹. Applicants respectfully disagree. Claim 1 recites “or a radical of general Formula II” (emphasis added) following “wherein R¹=,”. Additionally, in claim 1, the phrase is before the next wherein clause describing the alternatives for R². Furthermore, the specification describes “a radical of general Formula II” on page 5, lines 34-45, as a choice for R¹, not as an alternative to Formula I. Thus, from the language of claim 1 and from the specification, it is clear that “a radical of general Formula II” is one of the choices for R¹.

Additionally, the Examiner rejected claims 18 and 19 as indefinite for reciting “wherein two or more of the first, second and third sequences are selected from said group”. The claims have been amended without prejudice or disclaimer for clarification. The rejections as to claims 18 and 19 are believed to be rendered moot. Reconsideration and withdrawal of the rejections is respectfully requested.

Rejections Under 35 USC § 112, first paragraph

The Examiner rejects claims 1-20 under 35 U.S.C. § 112, first paragraph, for allegedly lacking an enabling disclosure. Applicants respectfully disagree and traverse the rejections. Nevertheless, in order to expedite prosecution, claims 1 and 12 have been amended without disclaimer or prejudice.

The Examiner alleges that the specification does not provide enablement for producing any and all compounds recited in claim 1 as having any "C2 to C4 alkylcarbonyl groups as R2 and R3". Applicants would like to clarify that, before the present amendment, claim 1 recited "C2-C24 alkylcarbonyl" not "C2-C4" (emphasis added). The amendment of claim 1 regarding C20 alkylcarbonyl groups is believed to render this rejection moot.

The Examiner also alleges that the specification does not provide enablement for transformation of any plant species with any set of three nucleic acids that may have as little as 50% identity with any of the recited SEQ ID numbers. The amendment of claim 12 regarding the percent identity is believed to render this rejection moot as well.

The Examiner further alleges that the specification only demonstrates expression of the delta-6 elongase of SEQ ID NO:3, delta-6 desaturase of SEQ ID NO:13, and delta-5 desaturase of SEQ ID NO:20 in tobacco and that the specification does not provide examples of any other compounds, of fatty acid profiles in other plants, or of transformation in other plants. Applicants respectfully disagree with the Examiner's characterization and conclusions.

First, compliance with the enablement requirement does not turn on whether an example is disclosed. See MPEP § 2164.02. There has never been a requirement that every species encompassed by a claim must be disclosed or exemplified in a working example. *In re Angstadt*, 537 F.2d 498 (CCPA 1976). Moreover, the specification provides several examples of constructs with multiple desaturases/elongases, for example, in Table 3 at page 47. The specification also provides examples of desaturases and elongases by GenBank Accession No. at page 47, lines 28-31, which can be used in the expression constructs.

Additionally, the specification exemplifies expression of a three gene construct in both tobacco and linseed, see Example 8, at page 53, lines 12-17. Also, a later publication by one of the inventors, when following the teachings of the present specification, describes several constructs with multiple genes and also exemplifies their expression in tobacco and linseed. (See Abbadi *et al.*, The Plant Cell, Vol. 16, 2004, pp. 2734-2748; a copy is attached for the Examiner's convenience). Abbadi *et al.* also discloses expression of a construct of the present specification in tobacco and linseed. *Id.* at page 2736, Figure 2, Construct C, for example. Abbadi *et al.* additionally discloses production of two C20 fatty acids in tobacco and four C20 fatty acids in linseed through expression of these genes (see Abbadi at page 2737, figure 4). The specification additionally provides guidance on identifying, isolating, and analyzing the genes or homologs of the genes of the invention as seen, for example, on page 30 line 15 through page 32 line 46, page 36 line 37 through page 40 line 2, examples 2-8. Thus, the specification not only provides examples, but sufficient guidance for one skilled in the art following the teachings of the specification, as evidenced in Abbadi *et al.*, for identifying genes, making expression constructs, and expression of the constructs resulting in the desired fatty acid profiles in several plant species.

The Examiner also alleges that sequence homology is not sufficient to predict function of fatty acid modification enzymes because computer analysis of genome sequences is flawed, citing to Doerks *et al.*, Smith *et al.*, Brenner *et al.*, and Bork *et al.* Applicants respectfully disagree.

The analysis in Doerks relates to assigning functional annotations to uncharacterized protein families (UPFs) that contain "putative, poorly annotated proteins that are usually labeled as 'hypothetical'." (Doerks, page 248, column 1, second paragraph, lines 2-5). These uncharacterized protein families differ from protein families such as the fatty acid modifying enzymes which have been cloned and functionally characterized in several different species. Even though Doerks selected uncharacterized protein families that were especially difficult to characterize, Doerks nevertheless discloses successful assignment of function to more than 700 of about 1300 proteins analyzed which were clustered in 25 of the 58 distinct UPFs by relating to proteins with annotated functional features. (See Doerks, page 250, first column, first full

paragraph, lines 1-4, and page 248, right column, second paragraph, last sentence). Furthermore, in the Doerks study, homologs were selected by PSI-BLAST based on an expected ratio of false positives ($E=0.001$) (see Doerks, page 248, middle column, third paragraph) and not by any specific level of sequence homology. The Examiner argues that Doerks states that the highest scoring database protein does not necessarily share the same functions (see Office Action page 4, citing to Doerks at page 248, first column, first paragraph, lines 20-23). However, the “highest scoring” protein in Doerks does not relate to a specific sequence homology and may actually share very little homology with the sequence of interest in contrast to the sequences of the present invention which share at least 50% (or 90%) homology. Doerks further discloses that when reasonable predictions could be made, the iterations were not continued. (see Doerks, page 248, left column, lines 10-13). In contrast to the Examiner’s conclusion, Doerks assigned and predicted function based on sequence comparisons to proteins with annotated functional features. (see Doerks, page 248, left column, lines 21-23)

Similarly in Brenner, the comparisons made were done without any specific sequence homology and were not done across sequence databases. The comparisons of Brenner were between three sets of functional annotations of the genes from a specific organism’s genome made by three groups of researchers. The Examiner alleges that Brenner states that most homologs must have different molecular and cellular function (see Office Action, page 4). However, this statement is out of context, rather Brenner states that “if there are only about 1000 major superfamilies in nature, then most homologs must have different molecular and cellular functions.” (Emphasis added, see Brenner, page 132, right column, second full paragraph, last sentence). Brenner makes this statement as a broad generalization based on extrapolating their findings of an 8% error rate in the comparisons of three annotations as being applicable to all public sequence databases (see Brenner, page 132, right column, first full paragraph, lines 4-5, and second paragraph).

Thus, because neither Doerks or Brenner show comparisons with specific sequence homology (in contrast to the sequences of the present invention which share at least 50% (or 90%) homology) and because the comparisons in Doerks and Brenner relate to a limited subset

of sequences (*i.e.* uncharacterized protein families and three annotations of one species genome), Doerks and Brenner are not applicable to support the Examiner's generalized conclusions.

The Examiner cites to Bork *et al.* for teaching numerous problems with sequences databases that can result in misinterpretation of sequence data. Bork *et al.* discuss factors such as sequencing errors, false translation of genomic DNA, spelling errors, and inconsistencies in gene nomenclature that result from automated data handling and analysis in large sequence databases. However, Bork *et al.* explain: "[m]any communities studying particular protein families or organisms know about specialized databases that contain much more information on particular genes or proteins, but which is often not linked to the major databases." (See Bork *et al.*, page 426, middle column, third paragraph, lines 9-14). Furthermore, Smith *et al.*, also cited by the Examiner, state "[c]learly, for researchers studying a particular protein family, most database annotation inconsistencies make little difference in the search for new, even distant members." (Smith *et al.*, page 1222, middle column, second paragraph). Therefore, both Bork *et al.* and Smith *et al.* acknowledge that many of the problems associated with large sequence databases are irrelevant or can be overcome when studying specific protein families such as with the protein families of the present application.

The Examiner further alleges that if sequences are identified only by similarity to other sequences that are known to encode fatty acid modifying enzymes, one cannot conclude that these sequences also encode fatty acid modifying enzymes, citing to Van de Loo, Broun *et al.*, and De Luca for unpredictability. The Examiner concludes that because of the lack of examples and the unpredictability it would require undue experimentation by one skilled in the art to make and use the invention as broadly claimed. Applicants respectfully disagree.

The Examiner bears the initial burden of showing nonenablement. See *In re Wright*, 999 F.2d 1557, 1561-62, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993). "Enablement requires that the specification teach those in the art to make and use the invention without 'undue experimentation.' . . . That some experimentation may be required is not fatal; the issue is whether the amount of experimentation required is 'undue.'" *In re Vaeck*, 947 F.2d 488, 495, 20 USPQ2d 1438, 1444 (Fed. Cir. 1991) (emphasis in original). Some experimentation, even a

considerable amount, is not “undue” if, e.g., it is merely routine, or if the specification provides a reasonable amount of guidance as to the direction in which the experimentation should proceed. See *In re Wands*, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988).

The Examiner argued that Broun *et al.* taught that a change in four amino acids converted a desaturase to a hydroxylase gene. However, Broun *et al.* supports enablement. The changes in the amino acids described in Broun *et al.* were in the conserved amino acid residues identified when the sequences of six desaturases and two hydroxylases were compared. Seven amino acid residues were found to be strictly conserved in the desaturases. (See Broun *et al.* page 1315, paragraph bridging center and right columns). The experiment described in Broun *et al.* resulted in “a major increase in desaturase activity and a decrease in hydroxylase activity upon introduction of the seven desaturase-equivalent residues into LfAH12,” a hydroxylase. (*Id.* at page 1316, left column, lines 26-32). Furthermore, modification of a single one of these seven conserved amino acids was found not to change the activity of the enzyme when compared to the wild type. (*Id.* at page 1316, right column, lines 1-4). Thus, Broun *et al.* actually teaches that a modification even in the conserved amino acid residues does not necessarily affect enzyme activity. The four amino acid residues (four of the seven conserved residues) cited by the Examiner were found adjacent to histidine residues that were identified as essential to catalysis. (*Id.* at page 1316, right column, last paragraph). Changes in all four of these specific amino acid residues adjacent to the active site did affect desaturase activity. Broun *et al.* concluded that because of the substantial effect of the four residues that abut the active site histidines, the difference between desaturase and hydroxylase was influenced by changes in active site geometry. (*Id.* at page 1317, left column, lines 22-27). Thus, the Examiner’s statements regarding identification of sequences by similarity to other known desaturases is inapposite to the teaching in Broun *et al.* Rather Broun *et al.* teaches which amino acids substitutions can and cannot be made which will affect enzyme activity from the identification of conserved residues based on sequence comparisons. Therefore, one of skill in the art when aligning various enzymes would readily be able to identify conserved residues and motifs, and, from the teachings of Broun *et al.* and the specification, know where and where not to effect substitutions.

Furthermore, in view of Examples 7 and 8, one skilled in the art would recognize that screening and testing for fatty acid modification activity in microorganisms and plant species is routine and is not undue experimentation. The same applies to screening and testing the activity of homologs of the sequences as claimed in the present application, which techniques are also known to one of skill in the art (see Broun *et al.* and Abbadi *et al.*). It is submitted that determining the functional activity is routine experimentation and not undue experimentation. Compare, *In re Wands*, 858 F.2d 731, 737 (Fed. Cir. 1988) (routine screening of hybridomas was not “undue experimentation;” the involved experimentation can be considerable, so long as “routine”). The test for whether experimentation is “undue” is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed to enable the determination of how to practice a desired embodiment of the invention claimed. *Ex parte Jackson*, 217 USPQ 804, 807 (1982).

In the present case, the specification provides detailed guidance and teaches in the Examples, as explained above, the types of routine assay which are employed to confirm the claimed activity and additionally working examples showing activity. For example, assembly of vectors for plant transformation is described in detail in Example 5 of the specification (page 42, line 7 to page 47, line 45). Methods of plant transformation are well known to one skilled in the art and are described in Example 4, (page 41, lines 19-45 to page 42 lines 1-5). Fatty acid analysis of transgenic seed is described in Example 8 (page 52, line 35 to page 53, line 3). On the facts of this case, the detailed guidance provided in the present specification and the routine nature of the screening for activity outweighs the unpredictability concerns noted by the Examiner. Some unpredictability in the art does not necessarily preclude enablement. Furthermore, the other *Wands* factors also weigh in Applicants’ favor, particularly “the state of the art” and “the relative skill of those in the art,” *In re Wands*, 858 F.2d 731, 736, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988), as evidenced by the prior art teachings (as seen in Broun *et al.*) and the specification.

In view of the detailed description, guidance, working examples, and high level of skill, the specification enables the full scope of the present claims without undue experimentation. On

these facts, an analysis under *In re Wands* supports enablement. *In re Wands*, 858 F.2d 731, 737 (Fed. Cir. 1988) (found that routine screening of hybridomas was not “undue experimentation;” the involved experimentation can be considerable, so long as “routine”).

For these reasons and in light of the amendments, reconsideration and withdrawal of this rejection is respectfully urged.

CONCLUSION

For at least the above reasons, Applicants respectfully request withdrawal of the rejections and allowance of the claims. If any outstanding issues remain, the Examiner is invited to telephone the undersigned at the number given below.

This response is filed within the three-month period for response from the mailing of the Office Communication, to and including April 28, 2008. Applicants believe no fee is due with this response. However, if a fee is due, please charge our Deposit Account No. 03-2775, under Order No. 12810-00043-US from which the undersigned is authorized to draw.

Respectfully submitted,

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Biosynthesis of Very-Long-Chain Polyunsaturated Fatty Acids in Transgenic Oilseeds: Constraints on Their Accumulation¹

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ω -6- and ω -3-polyunsaturated C20 fatty acids represent important components of the human diet. A more regular consumption and an accordingly sustainable source of these compounds are highly desirable. In contrast with the very high levels to which industrial fatty acids have to be enriched in plant oils for competitive use as chemical feedstocks, much lower percentages of very-long-chain polyunsaturated fatty acids (VLCPUFA) in edible plant oils would satisfy nutritional requirements. Seed-specific expression in transgenic tobacco (*Nicotiana tabacum*) and linseed (*Linum usitatissimum*) of cDNAs encoding fatty acyl-desaturases and elongases, absent from all agronomically important plants, resulted in the very high accumulation of Δ 6-desaturated C18 fatty acids and up to 5% of C20 polyunsaturated fatty acids, including arachidonic and eicosapentaenoic acid. Detailed lipid analyses of developing seeds from transgenic plants were interpreted as indicating that, after desaturation on phosphatidylcholine, Δ 6-desaturated products are immediately channeled to the triacylglycerols and effectively bypass the acyl-CoA pool. Thus, the lack of available Δ 6-desaturated acyl-CoA substrates in the acyl-CoA pool limits the synthesis of elongated C20 fatty acids and disrupts the alternating sequence of lipid-linked desaturations and acyl-CoA dependent elongations. As well as the successful production of VLCPUFA in transgenic oilseeds and the identification of constraints on their accumulation, our results indicate alternative strategies to circumvent this bottleneck.

INTRODUCTION

Very-long-chain polyunsaturated fatty acids (VLCPUFA) have 20 or 22 carbon atoms and 4, 5, or 6 methylene-interrupted cis-double bonds in ω -6- or ω -3-arrangements. The nutritionally most important VLCPUFA are arachidonic (ARA; ω -6-20:4^{5,8,11,14}), eicosapentaenoic (EPA; ω -3-20:5^{8,11,14,17}), and docosahexaenoic acid (ω -3-22:6^{7,10,13,16,19}). Human physiology depends in various ways on these VLCPUFA, either as components of membrane phospholipids in specific tissues or as precursors for the synthesis of the different groups of eicosanoid effectors (e.g., prostaglandins) (Jump, 2002). VLCPUFA are not only required for the development of the fetal neuronal system but also contribute via a multiplicity of beneficial roles to the maintenance of health with increasing development and age, particularly by reducing the incidence of cardiovascular diseases (Demailson and Moreau, 2002). These fatty acids are either

directly available as components of the diet or produced from the two essential ω -6- and ω -3-C18 fatty acids, linoleic (ω -6-C18:2^{9,12}) and α -linolenic acid (ω -3-C18:3^{9,12,15}) (Okuyama et al., 1996). Official organizations recommend that the intake of EPA should be 0.2 to 0.5 g per day (Trautwein, 2001). Thus, the daily consumption of \sim 10 g (i.e., a spoonful) of a plant oil with 2 to 5% of this fatty acid would meet the recommended dietary intake. By contrast, levels of so-called industrial fatty acids for use in the chemical industry have to represent \sim 90% of the fatty acids in seed oils to be considered commercially viable, which is 10 to 20 times higher than the nutritionally important proportion of VLCPUFA in edible oils.

Reliable dietary sources of VLCPUFA are fish oils, whereas linoleic and linolenic acid predominate in green vegetables and some plant oils, all of which do not contain VLCPUFA. On the other hand, most processed food items with extended shelf life are characterized by high proportions of saturated fatty acids and a very high ratio of ω -6/ ω -3-polyunsaturated C18 fatty acids, far above the recommended ratio of \sim 6:1 (Trautwein, 2001). Therefore, the fatty acid profiles of modern western diets represent a rather unfavorable situation for the endogenous biosynthesis of a balanced proportion of ω -6/ ω -3-VLCPUFA, particularly of EPA and docosahexaenoic acid (Sprecher, 2002). Because of the continuous decrease of marine resources by overfishing and the environmental impact of fish farming (Pauly et al., 2002; Hites et al., 2004), neither farmed nor caught

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fish can be considered a sustainable source of the VLCPUFA quantities required for a healthy nutrition of the growing human population. A solution to this predicted shortcoming may be realized by implementation of VLCPUFA biosynthesis into annual oilseed crops (Galili et al., 2002). This source would be more sustainable than production from single cell cultures of fungi or algae (Lopez, Alonso, and Garcia-Maroto, 2000). On the other hand, these microorganisms are the most efficient primary producers of VLCPUFA and express all the enzymes required for their synthesis from C18:2 and C18:3 (Beaudoin et al., 2000; Abbadi et al., 2001; Domergue et al., 2002).

Previous experiments to change the fatty acid profiles of plant reserve triacylglycerols (TAG) by seed-specific expression of various enzymes have shown that the outcome of these manipulations cannot be reliably predicted, and a central question remains as to the mechanism(s) by which unusual fatty acids are accumulated in TAG (Voelker and Kinney, 2001; Drexler et al., 2003a). For a conversion of the polyunsaturated C18 fatty acids into ARA or EPA, formally three consecutive enzymatic steps are required: $\Delta 6$ -desaturation, $\Delta 6$ -elongation, and $\Delta 5$ -desaturation (Figure 1). In a previous study, we demonstrated the reconstitution of ARA biosynthesis in yeast and showed that the front-end desaturases and elongases have strict acyl carrier specificities (Domergue et al., 2003). Here, we report experiments on the production of C20-PUFA in seeds of transgenic tobacco (*Nicotiana tabacum*) and linseed expressing appropriate heterologous enzymes under the control of seed-specific promoters and our effort to understand the channeling of acyl groups to TAG synthesis.

RESULTS

Engineering of ARA and EPA Biosynthesis in Transgenic Plants

In the following, we report experiments on the production of ARA and EPA in seeds of transgenic higher plants. As heterologous coding sequences we used cDNAs encoding two regio-specific

cally different desaturases ($\Delta 6$ and $\Delta 5$) and a $\Delta 6$ -elongase, each under the control of up to three different seed-specific promoters. The protein sequences were selected from a wide variety of VLCPUFA-producing organisms such as fungi (*Mortierella alpina*; Ma) (Michaelson et al., 1998), algae (*Phaeodactylum tricornutum*; P) (Domergue et al., 2002), mosses (*Physcomitrella patens*; Pp) (Säike et al., 1998; Zank et al., 2002), plants (*Borago officinalis*; Bo) (Sayanova et al., 1997), and lower animals (*Caenorhabditis elegans*; Ce) (Napier et al., 1998; Beaudoin et al., 2000). Four different combinations of these sequences were placed in binary vectors (Figure 2) and used for transformation of *N. tabacum* (high in linoleic acid, 18:2^{Δ5,12}) and *Linum usitatissimum* (high in α -linolenic acid, 18:3^{Δ5,12,15}) to identify a useful combination and a suitable expression host. For a first round of analysis, seeds of regenerated transgenic plants were collected at maturity and used for fatty acid analysis by gas-liquid chromatography (GLC)/mass spectrometry (MS).

Of the four combinations tested, only the expression of constructs C and D in tobacco and linseed were successful (Figure 3). Fifty independent transgenic tobacco and 46 independent linseed lines expressing the construct C were obtained by *Agrobacterium tumefaciens*-mediated transformation. The fatty acid compositions of pooled T2 seeds from a selection of transgenic lines (Figure 3) showed high proportions of the two $\Delta 6$ -C18-PUFAs γ -linolenic (18:3^{Δ6,9,12}) and stearidonic acid (18:4^{Δ6,9,12,15}) (2 to 29% in tobacco and 3 to 37% in linseed) as well as significant but disproportionately low levels of C20-PUFA (1 to 3% in tobacco and 0.5 to 8.3% in linseed; Figures 3A and 3B). These results indicate the following: (1) the promoters used enable sufficient expression in tobacco and linseed; (2) the plant enzymes (e.g. desaturases and moss elongase) seem to be more efficient than animal and fungal enzymes in the hosts selected; (3) to obtain significant proportions of C20-PUFA, the host plants should have high endogenous levels of linoleic and/or α -linolenic acid in their untransformed seeds.

Next, groups of the most promising linseed and tobacco transformants with the construct C were subjected to DNA gel blot analysis for a selection of plants with single copy integration to facilitate comparative studies and the generation of homogeneous T3 progeny for breeding. Fatty acid analysis of transgenic T3 seed mixtures showed that in tobacco the $\omega 6$ -pathway (Figure 4A) and in linseed both the $\omega 6$ - and $\omega 3$ -pathways (Figure 4B) were successfully reconstituted. Several new fatty acids were detected in the chromatograms of fatty acid methyl esters (FAMES) from transgenic tobacco and linseed, respectively (Figures 4A and 4B) that are not present in the control plants. Mass spectra of their 4,4-dimethyloxazoline derivatives identified them as γ -18:3^{Δ6,9,12}, 18:4^{Δ6,9,12,15}, 20:3^{Δ6,11,14} ($\omega 6$ -20:3), 20:4^{Δ6,9,11,14} ($\omega 6$ -20:4), 20:4^{Δ8,11,14,17} ($\omega 3$ -20:4, ARA), and 20:5^{Δ6,9,11,14,17} ($\omega 3$ -20:5, EPA) (data not shown). The fatty acid compositions of wild-type and transgenic seeds are given in Table 1. Among the new fatty acids produced in transgenic seeds, γ -18:3^{Δ6,9,12} is the most abundant and accounted for ~30 and 17% in tobacco and linseed, respectively. In linseed, 18:4^{Δ6,9,12,15} was the next most abundant fatty acid and accounts for ~13%. The presence of both γ -18:3^{Δ6,9,12} and 18:4^{Δ6,9,12,15} indicates that the $\Delta 6$ -desaturase from *P. tricornutum*

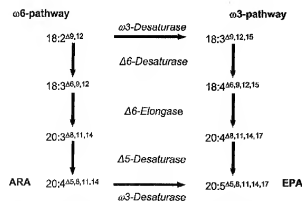


Figure 1. Simplified $\omega 6$ - and $\omega 3$ -Pathways for the Biosynthesis of VLCPUFA from Linoleic (18:2) and α -Linolenic (18:3) Acid, Respectively.

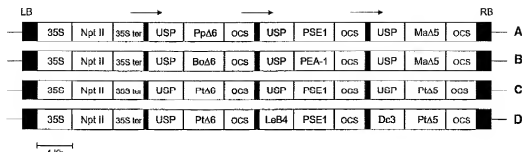


Figure 2. T-DNA of the Binary Vectors Used for Plant Transformation.

LB and RB, left and right T-DNA borders, respectively; USP, promoter region of the unknown seed protein of *Vicia faba*; LeB4, promoter of the legumin gene of *V. faba*; OCS, promoter of the helianthin gene of *Daucus carota*; OCS, terminator region of octoplin synthase gene of *A. tumefaciens*; 35S, 35S promoter of *Caulliflower mosaic virus* (CaMV); 35S ter, CaMV 35S terminator; Npt II, neomycin phosphotransferase gene; PpΔ6, BoΔ6, and PtaΔ6, Δ⁶-desaturases from *P. patens* (AJ222980), *B. officinalis* (J79010), and *P. tricornutum* (AY082393), respectively; MaΔ5 and PtaΔ5, Δ⁵-desaturases from *M. alpina* (AF054824) and *P. tricornutum* (AY082392), respectively; PSE1 and PEA-1, Δ⁶-elongase from *P. patens* (AF428243) and *C. elegans* (F56H11.4), respectively.

used both 18:2^{Δ5,12} and α-18:3^{Δ5,12,15} as substrates in plants. This leads to a decrease in 18:2^{Δ5,12} in transgenic tobacco and of 18:2^{Δ5,12} and α-18:3^{Δ5,12,15} in transgenic linseed. On the other hand, the various C20-PUFA fatty acids totaled 5%. ARA accumulated up to 2% in tobacco, whereas in linseed, both ARA (up to 1.5%) and EPA (up to 1%) were found (Figure 4, Table 1). The proportions of VLCPUFA in the T3 seeds were comparable to those found in the T2 progeny, indicating that a stable phenotype was transferred to the next generation. These data demonstrate that oilseeds can in fact be transformed to produce VLCPUFA and that the proportions we observed in linseed are already close to the nutritionally relevant levels described above.

For that reason, all the following experiments were conducted with linseed because of its importance as an oilseed crop.

Why Not More ARA and EPA in the Transgenic Seeds: Is the Elongase Step Limiting?

The high proportions of Δ⁶-desaturated C18-polyunsaturated fatty acids found in transgenic linseed (γ-18:3 and 18:4 totalling in some plants a maximum of 33%) contrasted with the low proportions of C20-PUFA, indicating that the elongating activity may limit the accumulation of C20-PUFAs. Using the same promoter in the construct C for the expression of all three genes

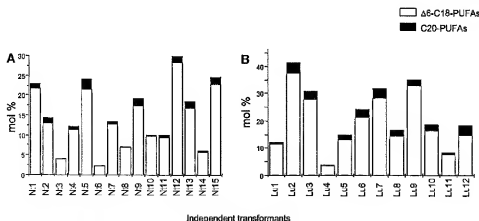


Figure 3. Δ⁶-C18- and C20-Polyunsaturated Fatty Acid Proportions in a Selection of Seeds from Primary Transforms of Tobacco and Linseed Transformed with Construct C.

Seeds were collected at 40 d after flowering (DAF) from transgenic tobacco (A) and linseed (B). FAMES were prepared from whole seeds and analyzed by gas chromatography (GC) as indicated in Methods. Open bars, Δ⁶-C18 polyunsaturated fatty acid proportions; closed bars, C20-polyunsaturated fatty acid proportions.

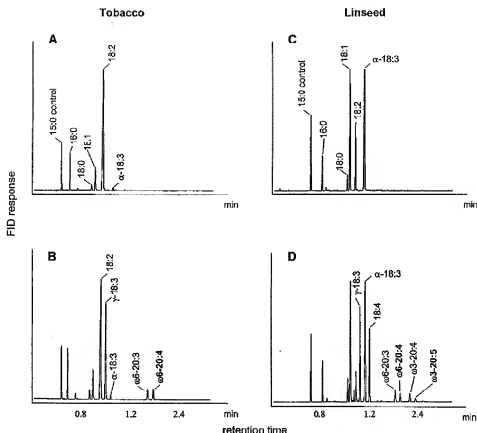


Figure 4. Fatty Acid Profiles of Seeds from Wild-Type and Single-Copy Tobacco and Linseed Transformants.

Seeds were collected at 40 DAF from wild-type ([A] and [C]) and transgenic ([B] and [D]) tobacco and linseed transformed with construct C (Figure 1). FAMEs were prepared from whole seeds and analyzed by GC as indicated in Methods. In this particular linseed plant, C20-polyunsaturated fatty acids accounted for 5% of the total fatty acids. It should be pointed out that none of the major peaks shown go off scale.

could conceivably induce gene silencing of one of the transgenes. To see whether the low elongation can be ascribed to reduced elongase transgene transcription in seeds, transcript levels of all three heterologous genes were tested by semiquantitative RT-PCR during three different stages of seed development. RT-PCR experiments showed that the three heterologous genes ($\Delta 6$ -desaturase, $\Delta 6$ -elongase, and $\Delta 5$ -desaturase) were transcribed at similar levels (data not shown). Furthermore, a nonradioactive assay has been developed to probe the elongase activity in total extracts and microsomes of the transgenic seeds. Incubation of microsomes from the transgenic seeds with 18:4-CoA as the elongase substrate without malonyl-CoA and reducing equivalents (NADH and NADPH) showed that 18:4-CoA was recovered after extraction of total acyl-CoAs (Figure 5A). By contrast, when 18:4-CoA was incubated in the presence of malonyl-CoA and reducing equivalents with microsomes, an additional peak corresponding to 20:4-CoA (coeluting with 18:2) appeared in the chromatogram of the extracted acyl-CoAs, indicating that 18:4-CoA has been

elongated efficiently to 20:4. A corresponding assay was conducted with γ -18:3-CoA. In this case, a similar extent of elongation resulted in the formation of 20:3-CoA (data not shown). According to these data, both the transcript levels and, hence, the enzyme activity of the elongase are unlikely to limit the accumulation of C20-PUFA. Therefore, we performed additional experiments to identify the cause(s) of this putative bottleneck.

Role of the Acyl-CoA Pool: Availability of $\Delta 6$ -Desaturated Fatty Acids Is Limiting

Recent studies with elongase and desaturases expressed in yeast suggest that plant and fungal desaturases (and in particular the front-end desaturases used in this approach) use phosphatidylcholine (PC)-linked acyl groups as actual substrates, whereas the elongase uses acyl-CoAs for condensation. Therefore, the desaturase will produce $\Delta 6$ -desaturated acyl groups as ester components of (phospho)lipids, whereas the $\Delta 6$ -elongase

Table 1. Fatty Acid Composition of Seeds from Wild-Type and Transgenic Tobacco and Unseeded Plants

Fatty Acid	Tobacco		Linseed	
	Wild Type	Transgenic	Wild Type	Transgenic
16:0	9.2 ± 0.2	9.9 ± 0.2	6.2 ± 0.5	5.5 ± 0.5
18:0	2.9 ± 0.1	3.1 ± 0.2	2.7 ± 0.3	3.8 ± 0.2
18:1 ^{AB}	11.8 ± 0.3	10.8 ± 0.3	30.8 ± 2.6	24.1 ± 1.7
18:2 ^{AB,19}	75.4 ± 1.2	43.6 ± 2.6	11.2 ± 0.6	5.6 ± 1.4
γ-18:3	ND	26.3 ± 1.7	ND	16.8 ± 2.8
α-18:3	0.6 ± 0.0	0.7 ± 0.1	49.0 ± 3.1	30.1 ± 3.2
18:4	ND	ND	ND	11.4 ± 2.3
ω6-20:3	ND	1.8 ± 0.2	ND	1.2 ± 0.4
ω6-20:4	ND	1.5 ± 0.3	ND	1.0 ± 0.5
ω3-20:4	ND	ND	ND	0.9 ± 0.3
ω3-20:5	ND	ND	ND	0.8 ± 0.2
ΣΔ6-C18-PUFAs		29.3 ± 1.7		28.2 ± 2.5
ΣC20-PUFAs		3.7 ± 0.5		3.6 ± 1.1

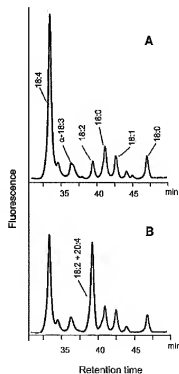
Seeds from wild-type and second generation progeny of single-copy transgenic tobacco and linseed were harvested at maturity, subjected to transesterification, and analyzed by GLC as indicated in Methods. Each value is the mean ± SD of seed mixtures from five independent plants. ND, not detected.

requires acyl-CoA thioesters as substrates (Domergue et al., 2003). For a better understanding of this aspect of metabolic interactions in transgenic linseed, we analyzed the acyl-CoA pools of untransformed and transgenic flax seeds at different developmental stages. After extraction, the intact thioesters were converted to their fluorescent etheno-derivatives and analyzed by HPLC. In the control thioester sample (Figure 6A), palmitic, stearic, oleic, linoleic, and α-linolenic acid were present, with palmitoyl-CoA being predominant. This profile differs from the fatty acid pattern of TAG mainly by the elevated proportions of palmitic (16:0) and stearic (18:0) acid. The occurrence of linoleic and α-linolenic acid in the acyl-CoA pool confirms previous data (Stymne and Stobart, 1984) describing the acyl-CoA:lysophosphatidylcholine acyltransferase (LPCAT) activity in microsomes from developing linseeds. On the other hand, the acyl-CoA sample from transgenic tissue (Figure 6B) differed only by the presence of trace levels of stearidonic acid during all stages investigated. This indicates either a very low exchange of 18:4 between the site of desaturation in PC and the acyl-CoA pool or a rapid removal of this fatty acid from the acyl-CoA pool. The same conclusion can be drawn for γ-18:3, which coelutes as a correspondingly small proportion in the α-18:3 peak. In addition, if we assume that linoleic and α-linolenic acid residues in lipids will be desaturated equally well by the Δ6-desaturase, then both should be equally well elongated by the Δ6-elongase after transfer into the acyl-CoA pool. However, in the acyl-CoA profile, we did not detect di-homo-γ-linolenic acid (ω6-C20:3), the elongation product of γ-C18:3, eluting between 16:0 and 18:1, elongated stearidonic acid (ω3-C20:4), or any other C20-PUFAs. Addition of various C18- and C20-PUFA-CoAs to seed extracts and microsomes from the transgenic plants and subsequent extraction of total acyl-CoAs indicate that the added acyl-CoAs are recovered by extraction and are not oxidized or subjected to

hydrolysis by an acyl-CoA thioesterase (Figure 5; data not shown). Therefore, the most obvious discrepancy to be explained in transgenic linseed is the following: Δ6-desaturated C18-PUFAs are found in high proportions in TAG, but they are almost completely absent from the acyl-CoA pool, whereas Δ9-unsaturated C18 fatty acids are present in roughly similar proportions in both TAG and the acyl-CoA pool. Such a distribution could be because of several mechanisms: (1) Δ6-C18-PUFAs are incorporated into TAG either via routes bypassing the acyl-CoA pool; (2) by highly efficient removal from the acyl-CoA pool giving the elongase no chance for a substantial conversion of its substrates; (3) by different subcellular compartmentation of desaturation and elongation reactions. Whatever the mechanism, the availability of Δ6-desaturated C18-CoA esters seems to prevent efficient reconstitution of VLCPUFA biosynthesis via the Δ6-elongating activity.

Channeling of VLCPUFA between Major Acyl Pools

As outlined above, all Δ6-desaturated C18-fatty acids have to be generated in PC as the primary site of desaturation before delivery into the acyl-CoA pool or channeling into TAG. To prove the role of PC in Δ6-C18- and in Δ5-C20-desaturation, we next

**Figure 5. In Vitro Assay of Elongase Activity.**

Microsomes from developing embryos were incubated with 18:4-CoA in the absence (A) and presence (B) of malonyl-CoA, NADH, and NADPH. After purification of the acyl-CoAs, they were converted to their etheno-derivatives and analyzed by HPLC. In the assay in (B), ~50% of 18:4-CoA is elongated to 20:4-CoA.

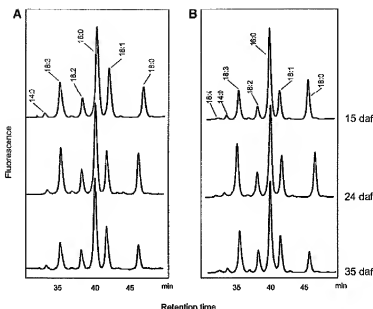


Figure 6. Profiles of Acyl-CoAs from Developing Wild-Type and Transgenic Linseed.

Immature seeds were harvested at 14, 24, and 35 DAF from wild-type (A) and transgenic (B) linseed expressing construct C and subjected to acyl-CoA extraction. The acyl-CoAs were converted to their etheno-derivatives and analyzed by HPLC.

analyzed the fatty acid profile of this central metabolite and compared it with representative components of lipid groups considered as receiving fatty acids or diacylglycerol (DAG) moieties from PC. For this purpose, the developing seeds at the stage of maximal lipid deposition (24 DAF) from wild-type and T3 transgenic linseed lines were subjected to extraction of lipids followed by separation into three classes: neutral lipids, glycolipids, and phospholipids (see Supplemental Table 1 online). This fractionation scheme was chosen to ensure that no significant component escaped detection. In all three classes, the fatty acid profiles of the transgenic samples differed from those of the wild type qualitatively only by the presence of $\Delta 6$ -C18-PUFA and C20-VLCPUFA, but a closer inspection reveals additional differences between the various lipid groups. γ -18:3 was nearly equally abundant in neutral lipids (15%), phospholipids (12%), and glycolipids (13%). On the other hand, 18:4 accumulated in neutral lipids (11%), whereas a reduction to equally low proportions is found in phospholipids (3.5%) and glycolipids (3%; see Supplemental Table 1 online). Furthermore, the proportion of the two $\omega 6$ -C20-fatty acids derived from γ -18:3 ($\omega 6$ -20:3 and $\omega 6$ -20:4) was twice as high in phospholipids (total 10%) as in neutral lipids (total 5%) and five times higher than in glycolipids (2%). By contrast, the two $\omega 3$ -C20-fatty acids derived from 18:4 ($\omega 3$ -20:4 and $\omega 3$ -20:5) were found in the neutral lipids (total 2.1%) but were hardly detected in phospholipids and glycolipids (see Supplemental Table 1 online). On the other hand, despite the fact that the $\omega 3$ precursor α -18:3 was five times more abundant than the $\omega 6$ precursor linoleic acid in wild-type linseed, the downstream products of the $\omega 3$ -pathway were

present in lower proportions than their $\omega 6$ counterparts in the transgenic plants. This unexpected observation encouraged us to conduct a more detailed analysis of the different lipid pools.

The three lipid fractions were used to purify TAG, DAG, two individual phospholipids (PC and phosphatidylethanolamine [PE]), and the two galactolipids monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (data not shown) because plastidial glycolipids of eukaryotic origin represent another pool of DAG derived from microsomal PC. Before considering the differences in fatty acid methyl esters between wild-type and transgenic samples (Figure 7), it is useful to compare in wild-type seeds the fatty acid profiles of PC with the two major lipid pools receiving fatty acids from PC: MGDG and TAG (Figure 7). It is obvious that α -18:3 is the predominant component in the accepting lipids MGDG and TAG, whereas the PC profile is dominated by 18:1, which originates from the plastid-derived acyl-CoA pool (Figure 7A). The lipids from transgenic seeds differ by the presence of $\Delta 6$ -C18-PUFA and C20-PUFA but reflecting to some extent the observations made with wild-type lipids. Figure 7B shows that 18:4 and $\omega 3$ -C20-PUFAs were concentrated in TAG contrasting with their low proportions in PC, DAG, PE, and MGDG. γ -18:3 was found nearly evenly distributed in all lipids. The major C20-PUFAs were $\omega 6$ -C20-PUFAs 20:3- $\omega 6$ and 20:4- $\omega 6$ (4.2 and 5.3%, respectively), which were present in much greater proportions than the two $\omega 3$ -C20-PUFAs and which were enriched in PC and DAG. γ -18:3 and 18:4 in MGDG (Figure 7B) must be considered as being of eukaryotic (i.e., microsomal) origin. In addition, very low proportions of $\omega 6$ -C20-PUFAs were also present. These results indicate that major

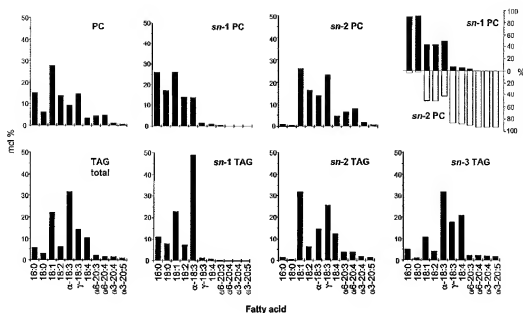


Figure 8. Regiospecific Distribution of Fatty Acids in PC and TAG from Developing Seeds of Transgenic Linseed (24 DAF).

The positional analysis of fatty acids from PC and TAG were determined as indicated in Methods. In all plots, except for the right PC plot, mol % refers to the percentage of each acyl group in the lipid class or at a particular position in that lipid class. In the right PC plot, "mol %" refers to the percentage of each fatty acid found in the sn-1 or sn-2 position. Each value is the mean from three independent experiments.

combined with an unsaturated C18-fatty acid. Unfortunately, we cannot extend this conclusion to γ -18:3 itself because the MS technique used does not differentiate between α - and γ -18:3.

Finally, we performed a stereospecific analysis of TAG from wild-type (see Supplemental Figure 1 online) and transgenic linseed, but only the data from the transformants are included in

Figure 8 for an immediate comparison with the profiles in equivalent PC positions. Our analysis of the wild-type TAG supports previous data (Matlouhi and Volpert, 1963) and shows the exclusion of saturated fatty acids from the sn-2 position but a more or less random distribution of 18:1 and 18:2 over all three positions, whereas 18:2 is enriched at the sn-2

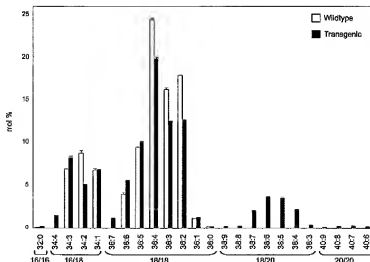


Figure 9. Molecular Species of PC from Developing Wild-Type and Transgenic Linseed (24 DAF).

position. By contrast, the three *sn*-positions of the transgenic TAG show different profiles. Confining the positional analysis to a lipase hydrolysis would not differentiate between *sn*-1 and *sn*-3, emphasizing the need for the more complicated analysis described here. The *sn*-1 profile is dominated by α -18:3, whereas 16:0 and 18:0 are significantly reduced when compared with the *sn*-1 position of PC. Δ 6-Desaturated fatty acids and their derivatives are nearly absent from this position paralleling the corresponding PC pattern. In the *sn*-2 profile, 16:0 and 18:0 are nearly absent, similar to the *sn*-2 position of PC. This similarity extends to the presence of γ -18:3 and 18:4 as well as to the C20 series. The proportion of γ -18:3 is highest in this position, but the two C20- ω 6-members (in contrast with the ω 3-members) are reduced compared with the PC pattern. Novortholose, the pair of *sn*-1 and *sn*-2 profiles of PC and TAG display some basic similarity. By contrast (and as might be expected from the complicated biochemical interactions to be discussed below), the acyl mixture at the *sn*-3 position of TAG differs most from all the others. It contains significantly less 16:0 and 18:0 than the *sn*-1 positions and also less 18:1 than any other TAG positions and the acyl donor pools (acyl-CoA thioesters, free DAG, PC-bound DAG, and its different *sn*-positions). The *sn*-3 profile is dominated by α -18:3 followed by significant and approximately equal proportions of 18:4 and γ -18:3. The four different C20 fatty acids are present in roughly equal proportions similar to the *sn*-2 profile but significantly different from the *sn*-2 position of PC with the characteristically uneven proportions of the C20- ω 6- and C20- ω 3-pairs.

Some of the important results of this stereospecific TAG analysis are the following: (1) all the new (i.e., non-native) C18 and C20 fatty acids are not present at the *sn*-1 position, (2) γ -18:3 is most abundant at the *sn*-2 position, and (3) by contrast, 18:4 is highest in the *sn*-3 position.

Taken together, these data show that the various pools (acyl-CoA thioesters, backbones, and the different *sn*-positions of PC, free DAG, galactolipids, and TAG) display fatty acid profiles with significant differences, in particular regarding the proportions of the heterologously expressed new fatty acids. This suggests the operation of selectivity and preference in the exchange of single acyl groups and/or complete DAG moieties between these pools. Thus, ω 3-C18- and ω 3-C20-PUFA are depleted in PC and efficiently moved to TAG. Compared with this, ω 6-18:3 and in particular its ω 6-C20-PUFA metabolites, appear to be effectively retained in the PC pool. In addition, a considerable proportion of 18:1, 18:2, and α -18:3 is moved through the acyl-CoA pool, whereas Δ 6-desaturated C18-PUFA and their elongation products can hardly be detected in this pool. The significance of these observations will be discussed in the next section.

DISCUSSION

The goal of our study was to produce transgenic plant oils enriched with VLCPUFA. The data presented demonstrate a successful reconstitution of C20-VLCPUFA biosynthesis in oil-synthesizing seeds of higher plants. The genetic basis of this implementation in linseed was the heterologous expression of three genes encoding a Δ 6-desaturase, a Δ 6-elongase, and a Δ 5-desaturase, the primary enzymes of VLCPUFA biosynthe-

sis. Of particular significance is the fact that, according to the dietary values recommended by nutritional organizations, the proportions of ~5% C20-PUFA reached in our transgenic seed oils would already be sufficient to satisfy these requirements. However, for expanded agricultural use, the current reported levels of ARA and EPA may still require some enhancement. On the other hand and perhaps of equal or greater significance, our data now offer new insights into the metabolic fluxes governing the production and deposition of these new fatty acids in seeds.

In previous experiments with the enzymes used in this study, we demonstrated that the Δ 6- and Δ 5-desaturase take place at the *sn*-2 position of PC and that the Δ 6-elongase uses acyl-CoA as substrates (Domergue et al., 2003). In addition, when expressed in yeast and supplemented with an exogenous fatty acid mixture reflecting that present in linseed, the moss Δ 6-elongase very efficiently elongated γ -C18:3 and 18:4, converting >50% of these substrates to C20-PUFAs (Zank et al., 2002). However, despite the accumulation of high proportions of Δ 6-desaturated C18-fatty acids in this study, our transgenic linseed lines did not use these substrates efficiently for elongation; therefore, the heterologous biosynthesis of VLCPUFA did not proceed significantly beyond the first desaturation step. Analysis of the acyl-CoA pool of the transgenic seeds at different developmental stages showed that γ -18:3- and 18:4-CoAs and their elongation products were hardly detectable, whereas 18:2- and α -18:3-CoA were present. This can be interpreted in two ways: either there was a selectively slow exchange of Δ 6-desaturated acyl groups between PC and the acyl-CoA pool, or the Δ 6-desaturated acyl groups were removed very rapidly from this pool by enzymes having higher affinity for these substrates than the elongase and simultaneously discriminating against 18:1, 18:2, and α -18:3. On the other hand, RT-PCR and the results presented in Figure 5 indicate that the elongase was transcribed correctly during seed development, resulting in high enzymatic activity during the main phase of lipid accumulation. Therefore, we assume that substrate availability rather than catalytic activity limited the elongation. This in turn suggests that additional mechanisms may contribute to the channeling of most of the Δ 6-C18-PUFA from PC to TAG by enzymes not dependent on the presence of these PUFA in the acyl-CoA pool. Our additional experiments were directed to understand these metabolic fluxes and to find out which of various alternatives contributed most to the acyl channeling in TAG synthesis.

Current understanding suggests that several reactions contribute to the flow of acyl groups between the various pools relevant in TAG biosynthesis, and some of them are in fact acyl-CoA independent (Figure 10). Several different mechanisms may be involved in the synthesis of DAG and TAG. DAG may be released from phosphatidic acid after *de novo* synthesis, but DAG may also be released from PC in the reverse cholinephosphotransferase (CPT) reaction. On the other hand, PC synthesis itself requires acyl-CoA-dependent *de novo* synthesis of DAG via phosphatidic acid. In addition, DAG may also result from an acyl-CoA-dependent acylation of monoacylglycerol (Beaudoin and Napier, 2004). The final conversion of DAG to TAG may be catalyzed by the acyl-CoA:DAG acyltransferase

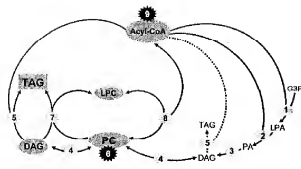


Figure 10. Accumulation of Linseed TAG by Channeling of Acyl Groups through PC.

The stepwise acylation of glycerol-3-phosphate (G3P) by acyl-CoA:1-glycerol-3-phosphate acyltransferase (G3PAT; 1) and acyl-CoA:1-acyl-glycerol-3-phosphate acyltransferase (2) yields 1-acyl-glycerol-3-phosphate (LPA) and phosphatidic acid (PA), which is hydrolyzed by phosphatidic acid phosphatase (3) to give DAG. Its conversion to TAG by acyl-CoA:diacylglycerol acyltransferase (5) immediately after the primary formation of DAG by the Kennedy pathway represents a minor alternative for TAG synthesis in linseed (indicated by the dotted arrow). The major part of DAG is converted to PC in a reversible reaction catalyzed by CDP-choline:diacylglycerol cholinephosphotransferase (CPT; 4). PC is the substrate for acyl group desaturases (6). They may accept both the *sn*-1 and *sn*-2-localized acyl groups (the genuine enzymes), or their action may be confined to the *sn*-2-bound acyl chains (the heterologously expressed $\Delta 6$ - and $\Delta 5$ -desaturases). In a reversible reaction, the acyl-CoA:lyso-phosphatidylcholine acyltransferase (8) equilibrates the *sn*-2-bound acyl group with the acyl-CoA pool. The formation of C20-polyunsaturated fatty acids by the heterologously expressed acyl-CoA elongase (9) occurs by elongation of $\Delta 6$ -C18-polyunsaturated acyl-CoA and thus depends on their release from PC into the acyl-CoA pool by the LPCAT (8). The activities of the desaturases (6) and elongase (9) together with the reversibility of LPCAT (8) and CPT (4) result in a continuous change of the acyl groups of the acyl-CoA pool, PC, and DAG, which all participate in this equilibration. Only after passage through PC, a highly desaturated DAG is finally converted into TAG. This channeling is catalyzed by two enzymes, the DAGAT (5) mentioned before and the phospholipid:diacylglycerol acyltransferase (PDAT; 7) that transfers the *sn*-2-bound acyl group from PC to the *sn*-3 position of TAG. These major routes, deduced by labeling studies with wild-type linseed (Slack et al., 1983; Stymne and Stobart, 1984), are supported by the acyl profiles of the various pools (acyl-CoA, PC, DAG, and TAG) studied in this investigation with transgenic linseed.

(DAGAT) (Voelker and Kinney, 2001) and by two acyl-CoA-independent reactions, for example, those catalyzed by the DAG:DAG transacylase (DDAT; not included in Figure 10) (Stobart et al., 1997) and the phospholipid:DAG acyltransferase (PDAT). This enzyme transfers the acyl-chain from the *sn*-2 position of PC to DAG yielding TAG and *sn*-2-lyso-PC (Dahlqvist et al., 2000). The acyl-CoA pool is fed mainly by acyl group export from plastids (16:0, 18:0, and 18:1) but changed in composition by the reverse reaction of the LPCAT (Stymne et al., 1983; Stymne and Stobart, 1984) by elongases and acyl-CoA synthetases. These activities result in a dynamic and heterogeneous acyl-CoA pool that is subject to further modification because of more or less selective removal by acyltrans-

ferases for de novo synthesis of phosphatidic acid and the deposition of acyl groups in TAG (Beaudoin and Napier, 2004). Thus, there are several acyl-CoA-independent routes by which PC-bound $\Delta 6$ - and $\Delta 5$ -desaturated acyl groups could be incorporated into TAG, explaining their absence from the acyl-CoA pool. The enzymes responsible for this channeling are CPT ($\Delta 6$ -desaturated acyl groups at least in the *sn*-2 position of TAG), DDAT ($\Delta 6$ -desaturated acyl groups at least in the *sn*-2 and *sn*-3 positions of TAG), and PDAT ($\Delta 6$ -desaturated acyl groups in addition in the *sn*-3 position of TAG).

Earlier studies (Slack et al., 1978, 1983; Somerville and Browse, 1991) suggested that in linseed the DAG and PC pools are essentially kept in equilibrium by the action of CPT. The major flux of glycerol and fatty acids incorporated into TAG proceeds predominantly via PC rather than depending on the stepwise use of 18:2- and 18:3-CoAs (derived from PC by exchange) for the uninterrupted and sequential formation of phosphatidic acid, DAG, and TAG via the classical Kennedy pathway. Taking into account that the $\Delta 6$ -desaturase is specific for the *sn*-2 position of PC and that $\Delta 6$ -C18-PUFA may not be efficiently exchanged from the *sn*-2 position of PC by the LPCAT, the DAG pool becomes enriched with the *sn*-2-bound $\Delta 6$ -C18-PUFA by conversion of PC to DAG by CPT. The fact that the fatty acid profile of total DAG resembled that of PC supports this assumption. These DAG molecules can then be incorporated into TAG by the action of DAGAT, PDAT, and DDAT or serve as substrates for the synthesis of other phospholipids (PE) and of glycolipids in the plastids (Figure 10).

On the other hand, the low level of 18:4 in PC and DAG and its abundance in TAG suggests that after desaturation this fatty acid is immediately shuttled to TAG. A possible mechanism for this channeling could be a high activity of the PDAT enzyme in addition to the channeling via the CPT-catalyzed release of DAG backbones. Positional analyses of PC and TAG from developing seeds indicate that γ -18:3 and 18:4 were absent from the *sn*-1 positions of both lipids. γ -18:3 was confined to the *sn*-2 position in PC and found at the *sn*-2 and *sn*-3 positions of TAG with some preference for the *sn*-2 position. By contrast, 18:4 was found to accumulate preferentially at the *sn*-3 position of TAG (Figure 8). The enrichment of 18:4 at the *sn*-3 position of TAG can be explained in two ways. One possibility is that the DAGAT prefers 18:4-CoA as substrate to form TAG rich in this fatty acid. This could explain the observation that in developing transgenic seeds 18:4-CoA is hardly detected in the acyl-CoA pool. An alternative possibility is that a PDAT-like enzyme is responsible for the direct channeling of 18:4 from the *sn*-2 position of PC to the *sn*-3 position of TAG. This alternative would be in line with the fact that 18:4 is a major component in the *sn*-3 position of TAG, but only a minor component in PC, absent in the acyl-CoA pool and only marginally elongated.

At this point it is interesting to compare our data on the positional distribution of $\Delta 6$ -desaturated fatty acids in transgenic linseed with those determined previously in TAG from two transgenic Brassica species and the well known γ -18:3-rich oils (Gunstone, 1992) from evening primrose (*Oenothera biennis*), black currant (*Ribes nigrum*), and borage (*B. officinalis*). The last three plants direct γ -18:3 into the *sn*-2 and *sn*-3 positions with only very minor proportions in *sn*-1. The $\Delta 6$ -desaturases of these

plants also seem to be specific for the *sn*-2 position of PC (Stymne and Stobart, 1986; Domergue et al., 2003). In transgenic *Rassia juncea* expressing a $\Delta 6$ -desaturase from *Pythium irregulare*, γ -18:3 produced in the seeds was not found in polar lipids, but almost all of it was targeted preferentially to the *sn*-2 position of TAG, whereas 18:4 was equally present in *sn*-2 and combined (*sn*-1 + *sn*-3) positions (Hong et al., 2002). In the related species *B. napus*, coexpression of $\Delta 12$ - and $\Delta 6$ -desaturases from *M. alpina* resulted also in a high accumulation of γ -18:3 in TAG. But in this case three-quarters of this fatty acid were located at *sn*-1 + *sn*-3 positions (not differentiated) of the TAG (Liu et al., 2001). These unexpected differences demonstrate that even among closely related plant species different mechanisms do occur for the channeling of $\Delta 6$ -desaturated acyl groups into TAG.

A further complication results from the fact that TAG synthesis occurs in parallel to phospholipid synthesis in the endoplasmic reticulum rather than in a separate compartment such as the differentiated oil body itself (Beaudoin and Napier, 2004). Therefore, the confinement of specifically desaturated fatty acids to TAG has to be ascribed to the specificity of the enzymes mentioned above, which in addition may prefer (or be associated with) pools of specific molecular species. Whether

this could be because of a lateral heterogeneity of endoplasmic reticulum membranes is still an open question (Vogel and Browse, 1996). Our analysis of the molecular species of PC from wild-type and transgenic seeds (24 DAF) indicates that the C20 fatty acids in the transgenic samples are found to be primarily combined with C18 fatty acids rather than with 16:0. These species seem to be precursors for the enrichment of C20 fatty acids in TAG. Therefore, the different pools of PC may not contribute to the same extent to TAG synthesis and its enrichment with polyunsaturated fatty acids. This has been suggested in previous studies dealing with plants accumulating polyunsaturated fatty acids (Roughan and Slack, 1982; Beaudoin and Napier, 2004).

Our data with linseed indicate that after desaturation, $\Delta 6$ C18-PUFA are likely to be channeled from PC directly to other lipids, preventing their exchange with the acyl-CoA pool and efficient subsequent elongation. Accordingly, the introduction of lipid-linked $\Delta 6$ -desaturation products into the acyl-CoA pool represents the most severe bottleneck for the production of VLCPUFA in transgenic linseed. A possible candidate enzyme regulating the above mentioned bottleneck (i.e., making available the $\Delta 6$ -desaturated C18-PUFA for elongation) may be LPCAT (Stymne and Stobart, 1984). In linseed cotyledons, this enzyme was

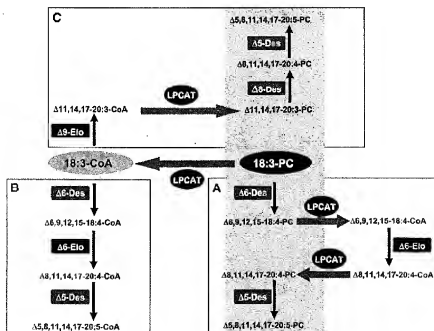


Figure 11. Differences in the Interplay between Desaturases, Elongases, and Acyltransferases in the Biosynthesis of VLCPUFA in Transgenic Plants.

Three different strategies can be used to generate VLCPUFA.

(A) The lipid-linked desaturation pathway was followed by our approach and requires both forward and reverse reactions of a $\Delta 6$ -specific LPCAT, which is the limiting step in linseed.

(B) The acyl-CoA pathway, where both the elongation and desaturation occur in the acyl-CoA pool, requires acyl-CoA front end desaturases so far only known from mammals. PC, phosphatidylcholine; Elo, elongase; Des, desaturase; light gray shading represents the membrane environment.

(C) An alternative pathway relying on the initiating $\Delta 6$ -elongation step in the acyl-CoA pool followed by lipid-linked desaturations has been verified in leaves (Qi et al., 2004).

shown to discriminate against saturated fatty acids, whereas 18:2 and α -18:3 produced in PC were rapidly returned to the acyl-CoA pool (Stymne and Stobart, 1984). Our new data confirm this observation and can be interpreted as showing that the linseed LPCAT enzyme may discriminate in addition against non-native Δ 6-polyunsaturated fatty acids, in particular for the initially slower reverse (PC to acyl-CoA) reaction (Stymne et al., 1983). At present, we do not know whether the same activity in different oilseed crops displays a similar selectivity. However, we would expect a significant increase in Δ 6-elongation by the coexpression in linseed of a LPCAT activity accepting Δ 6-desaturated C18 fatty acids.

Strategies to Improve VLCPUFA Synthesis in Transgenic Plants

The identification of the bottleneck in our approach raises additional interest in alternative strategies for VLCPUFA biosynthesis, as briefly outlined here (Figure 11). For example, the alternative shown in Figure 11B makes use of animal Δ 6- and Δ 5-front end desaturases that have been shown to use acyl-CoA as substrates (Okayasu et al., 1981; Irazu et al., 1993). With such enzymes, the conversion of linoleic or α -linolenic acid to ARA or EPA would be accomplished exclusively on the acyl-CoA track and thus avoid the switching between lipids and acyl-CoA (Figure 11B). Analyses of acyl-CoA profiles in developing seeds from linseed (our data) and rapeseed (Larson et al., 2002) have shown that this pool is indeed nearly in equilibrium with regard to the primarily lipid-linked Δ 9-polyunsaturated desaturation products, such as linoleoyl and α -linolenoyl residues.

The abundance of these Δ 9-polyunsaturated C18 fatty acids in plant acyl-CoA pools suggests that the bottleneck observed in the lipid-linked Δ 6-desaturation/ Δ 6-elongation sequence (Figure 11A) could be circumvented by yet another alternative depicted in Figure 11C. It depends on the sequential action of a Δ 9-elongase and a Δ 8-desaturase. cDNA sequences for both enzymes have been cloned from algae (Wallis and Browne, 1999; Qi et al., 2002). On expression in developing seeds this Δ 9-elongase would have access to the pool of Δ 9-C18-polyunsaturated acyl-CoAs and produce Δ 11-C20-polyunsaturated acyl-CoAs, which after a presumed incorporation into PC would become substrates for the subsequent desaturation by the Δ 8-desaturase forming lipid-linked Δ 9-C20-PUFA. They in turn would serve immediately as substrate for a lipid-linked Δ 5-desaturase. This Δ 9-elongation/ Δ 8-desaturation sequence requires an additional acyltransferase (LPCAT) for incorporation of the Δ 11-C20-PUFA into phospholipids to make them acceptable by the Δ 8-desaturase. This forward acylation of lyso-phospholipids may be less selective than the reverse reaction required for releasing lipid-bound acyl groups into the acyl-CoA pool. Recently, a successful implementation of this strategy in nonseed tissues of *Arabidopsis thaliana* led indeed to the accumulation of C20-PUFAs in leaves (Qi et al., 2004). Whatever strategy used, the selectivity of a LPCAT seems to play a critical role in the successful reconstitution of the conventional C20-PUFA biosynthetic pathway.

Further efforts will be directed toward increasing the levels of C20-PUFAs reported in this study. However, our data clearly

demonstrate the feasibility of the production of these nutritionally important fatty acids in transgenic oilseeds. A supporting approach might be to produce similar levels of these beneficial fatty acids in green vegetables, which are particularly rich in the precursor α -18:3 (Qi et al., 2004). The availability of such oils and vegetables enriched in VLCPUFA will be a new addition to the list of functional food items of plant origin designed for increased levels of minor, but nutritionally highly relevant, components such as β -carotenes (Ye et al., 2000), tocopherols (Shintani and DellaPenna, 1998), and vitamin C (Agius et al., 2003).

METHODS

Materials

Restriction enzymes, polymerases, and DNA modifying enzymes were obtained from New England Biolabs (Frankfurt, Germany). *Linum usitatissimum* (cv Flanders) was obtained from CECECO (Vlijmsen, The Netherlands). All components for plant media were obtained from Duchefa (Haarlem, The Netherlands). The *Agrobacterium tumefaciens* strain C58C1 ATHV, a derivative of strain EHA 101 (Hood et al., 1986), was kindly provided by J. Dettondörfer (KWS, Eibbeck, Germany). All other chemicals were from Sigma (St. Louis, MO) unless indicated otherwise.

Plant Transformation Constructs

The binary vectors pCAMBIA2300 (CAMBIA, Canberra, Australia) and pGPTV (A251014), which both use the *nptII* gene with the CaMV 35S promoter as the selectable marker, were used for the construction of plasmids for plant transformation. For the generation of constructs A, B, and C (Figure 2), a triple cassette containing the USP promoter (Bäumlein et al., 1991), the OCS terminator, and three different polylinkers between each promoter and terminator were first inserted into the pUC19 vector (Pharmacia, Erlangen, Germany), yielding the USP123OCS plasmid. The open reading frames of the different desaturases and elongases were modified by PCR to create appropriate restriction sites adjacent to the start and stop codons, cloned into the pKilM1-1 vector (Promega, Madison, WI), and sequenced to confirm their identity. The open reading frames were then released using the restriction sites created by PCR and successively inserted into the same sites of the polylinkers of the USP123OCS plasmid. The resulting cassette, containing the three genes each under the control of the USP promoter, was released by digesting the USP123OCS plasmid with *AscI* or *EcoRI* and *HindIII* and cloned into the corresponding sites of the binary vectors pGPTV and pCAMBIA2300, respectively. For the generation of construct D (Figure 2), the USP promoter at the second and third positions of the cassette in the USP123OCS plasmid was replaced by the LeB4 promoter (Fisdor and Conrad, 1995) and the Do3 promoter (Thomas, 1993), respectively. The corresponding plant construct was then generated as described above. Plasmid DNA from all constructs was isolated and purified according to standard methods. All vectors were transferred into the *Agrobacterium* strain C58C1 ATHV via electroporation.

Plant Transformation

Linseed transformation was performed according to Draxler et al. (2003b). Regenerated shoots were selected on MS medium with 1.5% (w/v) sucrose, 400 mg/L of carbenicillin, and 60 mg/L of G418. After rooting they were transferred to pots and grown at 22°C under 16-h daylight in a growth chamber. The flowers were labeled at anthesis, and immature and mature seeds were harvested at 24 and 40 d after flowering

(DAF), respectively. The tobacco (*Nicotiana tabacum*) transformation was conducted as described previously (Jongsma et al., 1995).

Fatty Acid Analysis

Ten milligrams of seeds from wild-type and transgenic plants were used for preparation and analysis of fatty acid methyl esters as described before (Domergue et al., 2002). For the analysis of immature seeds, seed probes were used to prepare parallel samples of cell debris, the wash fraction, and the acyl-CoA extract (see below), which were subjected to acid transesterification and analyzed by GLC as described (Domergue et al., 2002). FAMES were identified by comparison with appropriate reference substances and by GLC/MS of their 4,4-dimethylxazoline derivatives as described previously (Sperling et al., 2000).

Preparation of Cell-Free Homogenates and Microsomes

Transgenic embryos (20 to 24 DAF, 100 to 200 mg fresh weight) were isolated at 0°C and homogenized in grinding medium (100 mM Hepes buffer, 0.32 M sucrose, 0.1% BSA, and 10 mM 2-mercaptoethanol, pH 7.2). After filtration through Miracloth (Merck, Darmstadt, Germany), protein was estimated according to Bradford (1976), and an aliquot of the cell-free homogenate was directly used for assaying elongation activity. Microsomes were prepared as indicated by Dahlqvist et al. (2000).

Assay of Elongation Activity

An aliquot of the cell-free extract (200 μ L, 200 μ g protein) or microsomes (500 μ L, 150 μ g protein) was incubated at 30°C for 1 h with 90 mM Hepes, pH 7.2, 10 mg/mL of BSA, 1 mM ATP, 0.5 mM NADH, 0.5 mM NADPH, 2 mM $MgCl_2$, 100 μ M malonyl-CoA, and 23.2 μ M 18:4-CoA in a final volume of 1 mL. The reaction was stopped by adding 200 μ L of 3 M acetic acid and 25 μ L of a saturated $(NH_4)_2SO_4$ solution. The mixture was briefly centrifuged, and the acyl-CoA fraction was purified on a Sep-pak column (Strata C18-E; Phenomenex, Torrance, CA) using acetonitrile as the eluting solvent. The samples were dried under argon and derivatized as indicated below.

Acyl-CoA Analysis

Saturated and monounsaturated acyl-CoA esters with acyl chain lengths from C12 to C18 were obtained from Sigma. Polyunsaturated acyl-CoAs (18:2n-6, 18:3n-7, 18:4n-2, 20:2n-6, 20:3n-3, 20:4n-6, and 20:4n-3-CoAs) were synthesized enzymatically using an acyl-CoA synthetase from *Pseudomonas* sp (Sigma). The reaction mixture (200 μ L) containing 100 mM Tris-HCl, pH 8.1, 10 mM $MgCl_2$, 5 mM ATP, 5 mM CoASH, 2 mM DTT, 25 μ M free fatty acid, and 2.5 units of acyl-CoA synthetase was incubated at 37°C for 2 h. The reaction was stopped with 50 μ L of glacial acetic acid/ethanol 1:1 (v/v), and the samples were washed with petroleum ether to extract residual free fatty acids. After purification of the acyl-CoAs on a Sep-pak column (Strata C18-E; Phenomenex) using acetonitrile as the eluting solvent, the samples were dried under argon and dissolved in 50 mM Mes, pH 5.0.

Acyl-CoA analysis was performed as described by Larson and Graham (2001) with slight modifications: acyl-CoAs were extracted from 20 mg of immature seeds (24 DAF) and washed with petroleum ether. The resulting extract was dried under argon at 50°C. Chloroacetaldehyde reagent (300 μ L) was then added to the samples, and the derivatization of acyl-CoAs to their etheno derivatives was conducted at 85°C for 20 min.

HPLC analysis was performed with Thermoquest HPLC equipment (Thermoquest, Eggenstein, Germany) using a LUNA 150 \times 2.0 column with phenyl-hexyl coated 5- μ m silica particles (Phenomenex) under the same conditions as described by Larson and Graham (2001).

Lipid Analysis

Lipid extracts from developing seeds of wild-type and transgenic plants were prepared as follows. Between 0.5 and 1 g of seeds were heated in water at 90°C for 2 min and homogenized in 15 mL of chloroform/methanol (1:2). The lipids were extracted on a shaker for 4 h and then for 20 h with 15 mL of chloroform/methanol (2:1). The resulting organic phases were combined and then extracted with 9 mL of 0.45% NaCl, dried with Na_2SO_4 , and evaporated under vacuum. The residue was dissolved in 1 mL of chloroform/methanol (2:1) representing the total lipid extract. Separation of lipid classes (neutral lipids, glycolipids, and phospholipids) was achieved using a Sep-pak column. Lipid extracts were loaded on the Sep-pak column pre-equilibrated with chloroform and then fractionated into the three lipid classes by elution as follows: neutral lipids with chloroform, glycolipids with acetone/isopropanol (8:1), and phospholipids with methanol. The isolation of individual components from the different lipid classes was achieved by HPTLC using a horizontal development tank (Camag, Berlin, Germany) with appropriate running solvents and standards.

Regiospecific Analysis of PC and TAG

Positional analysis of PC was conducted using the *Rhizopus arrizus* lipase from Sigma as described previously (Domergue et al., 2003). The regiospecific analysis of TAGs was based on the partial degradation with Grignard reagent according to Becker et al. (1993) except that ethyl magnesium bromide (Merck, Rahway, NJ) was used instead of allyl magnesium bromide. For this purpose, 30 mg of purified TAG were dissolved in diethyl ether (1.4 mL) in a glass tube, and ethyl magnesium bromide (3 M in 100 μ L of diethyl ether) was added. After 1 min of vigorous agitation, the reaction was stopped by addition of 4 mL of an acidic buffer (1 volume of 37% HCl and 36 volumes of 0.4 M boric acid solution in water). Five milliliters of diethyl ether were added, and the organic phase was washed twice with 0.4 M boric acid. After discarding the aqueous phase, the organic phase was dried with Na_2SO_4 and evaporated to dryness under argon without heating to avoid isomerization of the DAG. The residue was dissolved in chloroform and separated by HPTLC using chloroform-acetone (96:4 [v/v]) as solvent. The zone corresponding to 1,2-DAG and 2,3-DAG (unresolved) was scraped off the plate and extracted from the silica with chloroform/methanol (2:1 [v/v]), dried under argon without heating, and dissolved in 1 mL of anhydrous pyridine (Merck). The DAG mixture was converted to phosphatidylphenol derivatives by incubation with 100 μ L of phenyldichlorophosphate (Merck) overnight at room temperature. Phosphatidylphenols were extracted according to Hajra (1974) and purified by HPTLC using chloroform/methanol/water (70:20:3 [v/v]) as solvent. After extraction from the silica (Hajra, 1974), the residue was evaporated to dryness, dissolved in 0.5 mL of diethylether, and sonicated four times for 20 s. Positional analysis was then conducted with a phospholipase A_2 from bee venom (Sigma) by adding 1 mL of buffer (0.1 M boric acid, 5 mM $CaCl_2$, and 0.1% Triton X-100, pH 8.9) and 10 μ L of phospholipase A_2 . The mixture was incubated at 25°C for 8 h under agitation to ensure completeness of the reaction. Lipolysis products were separated by HPTLC using as solvent chloroform/methanol/aqueous ammonia (65/5/5). The spots corresponding to free fatty acids (*sn*-2), lyso phosphatidylphenols (*sn*-1), and 2,3-phosphatidylphenols were subjected directly to transesterification and GC analysis. Reincubation of the recovered 2,3-phosphatidylphenols with phospholipase A_2 did not result in any *lysn*-compounds, indicating complete hydrolysis of 1,2-phosphatidylphenols. The distribution of fatty acids in the *sn*-3 position of TAG was deduced using the following formula: $sn\text{-}3\text{ (mol \%)} = [3 \times TAG\text{ (mol \%)}] - [sn\text{-}1\text{ (mol \%)}] - [sn\text{-}2\text{ (mol \%)}]$
 $sn\text{-}3\text{ (mol \%)} = [2 \times 2,3\text{-acyl-}sn\text{-phosphatidylphenol (mol \%)}] - [sn\text{-}2\text{ (mol \%)}]$

Analysis of PC Molecular Species

Molecular species analysis of PC was performed by electrospray tandem MS by scanning the precursors of the ions with the mass-to-charge ratio 184 in the positive mode, as previously described (Wells et al., 2002). Analyses of the acyl species in the PC species of each mass were performed by analyzing the acyl ion products of the acetate anions of the PC species (Wells et al., 2002).

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under the following accession numbers. $\Delta 6$ -desaturases: Pp $\Delta 6$ (AJ222980), Bo $\Delta 6$ (U79010), and Pt $\Delta 6$ (AY082393). $\Delta 5$ -desaturases: Ma $\Delta 5$ (AF054824) and Pt $\Delta 5$ (AY082392). $\Delta 6$ -elongases: PSE1 (AF428243) and PEA-1 (AX035537).

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